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Molecular cloning, expression, and distribution of glomerular epithelial protein 1 in developing mouse kidney

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Molecular cloning, expression, and distribution of glomerular epithelial protein 1 in developing mouse kidney.

Background. Glomerular epithelial protein 1 (GLEPP1) is a receptor-like membrane protein tyrosine phosphatase (RPTP) with a large ectodomain consisting of multiple fibronectin type III repeats, a single transmembrane segment, and a single cytoplasmic phosphatase active site sequence. In adult human and rabbit kidneys, GLEPP1 is found exclusively on apical membranes of podocytes and especially on surfaces of foot processes. Although neither ligand nor function for this protein is known, other RPTPs with similar topologies have been implicated in mediating adherence behavior of cells.

Methods. To evaluate potential roles of GLEPP1 further, we cloned the full-length mouse GLEPP1 cDNA and examined its expression patterns in developing kidney by Northern blot analysis, in situ hybridization, and immunofluorescence microscopy.

Results. Nucleotide sequencing showed that mouse GLEPP1 was approximately 80% identical to rabbit and human GLEPP1 and approximately 91% identical at the amino acid level. The membrane-spanning and phosphatase domains of mouse GLEPP1 shared >99% homology with PTPϕ, a murine macrophage cytoplasmic phosphatase. Northern analysis identified a single GLEPP1 transcript of approximately 5.5 kb in fetal kidney that became approximately threefold more abundant in adults. In situ hybridization of newborn mouse kidney revealed GLEPP1 mRNA in visceral epithelial cells (developing podocytes) of comma- and S-shaped nephric figures, and expression increased in capillary loop and maturing stage glomeruli. Beginning on embryonic day 14, GLEPP1 protein was first observed on cuboidal podocytes of capillary loop stage glomeruli, but nascent podocytes of earlier comma- and S-shaped nephric figures were negative. At later stages of glomerular maturation, where foot process elongation and interdigitation occurs, GLEPP1 immunolabeling intensified on podocytes and then persisted at high levels in fully developed glomeruli.

Conclusion. Our findings are consistent with a role for GLEPP1 in mediating and maintaining podocyte differentiation specifically.

Key words: glomerulus, podocyte, phosphatase, kidney development, cell function.

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The phosphorylation of protein serine/threonine/tyrosine residues by kinases and dephosphorylation by phosphatases mediate many cellular functions, including adhesion, proliferation, and differentiation [1, 2]. Protein tyrosine phosphatases (PTPs) represent a structurally diverse family of both cytoplasmic (soluble) and receptor (membrane-associated) forms, and more than 100 PTPs from yeast to vertebrates, as well as in some bacterial species, have been cloned to date [1, 3–5]. All PTPs share a highly conserved phosphatase catalytic domain of approximately 240 amino acid residues, in which there is an 11-residue signature sequence [(I/V)HCXAGXGR (S/T)G]. Within this unique motif, the cysteine residue is essential for the specific catalytic activity [4, 6].

The receptor PTPs (RPTPs) are composed of one or two tandem phosphatase domains intracellularly, a single transmembrane segment, and variable extracellular domains. One or more tandem repeats of fibronectin type III and immunoglobulin-like motifs are common in the extracellular domain, whereas MAM-(meprin/A5/PTPμ) and carbonic anhydrase-like motifs have also been reported [7–9]. The ectodomains of many RPTPs also have strong sequence homologies to cell adhesion molecules and to extracellular matrix proteins, suggesting an involvement in cell–cell and/or cell–matrix interactions [10–12]. Both homophilic and heterophilic interactions between ectodomains of RPTPs and their ligands have been shown. For example, the overexpression of extracellular segments of RPTPμ and RPTPκ on the surface of insect cells leads to homotypic interactions between the RPTPs [13, 14]. On the other hand, RPTPβ has been found to interact with the adhesion molecules N-CAM and Ng-CAM, the extracellular matrix protein tenascin, and the neuronal cell surface molecule contactin [15–17].

Glomerular epithelial protein 1 (GLEPP1), which belongs to the fibronectin type III RPTP family, is found only in the kidney and brain and in the kidney localizes specifically to the apical cell membranes of glomerular podocyte foot processes [18, 19]. Although neither the ligand nor function of GLEPP1 has been defined, some studies suggest that it might act as a sensor for visceral glomerular epithelial cells foot process structure and/or function [20]. In both human glomerular disease and in animal models of glomerulonephritis, the expression levels for GLEPP1 mRNA and protein are markedly reduced early during foot process effacement, which also suggests that GLEPP1 might become a useful clinical marker for glomerular injury [21].

As a glomerular podocyte-specific RPTP, GLEPP1 may therefore be a candidate molecule important for establishing and regulating the unique morphologic and functional characteristics of that cell. To help understand the function of GLEPP1 in more detail, we have cloned its full-length cDNA from the mouse and have investigated the expression of this protein during murine kidney development.

METHODS

RNA preparation and library construction

Kidneys were removed on the 15th gestational day from ether-anesthetized CD-1 pregnant mice, newborn (1 day old), and six-week-old mice and were immediately homogenized in ice-cold 4 mol/L guanidine isothiocyanate buffer [22]. Homogenates were layered over 5.7 mol/L CsCl, and total RNAs were obtained by ultracentrifugation and ethanol precipitation. Total RNA harvested from newborn mouse kidneys was used to construct a λgt11 cDNA expression library (Clontech Laboratories, Inc., Palo Alto, CA, USA) using both oligo(dt) and random primers.

Polymerase chain reaction-based cDNA cloning

The full-length murine GLEPP1 cDNA was isolated using a combination of polymerase chain reaction (PCR) amplification of the newborn mice kidney λgt11 library and reverse transcription-PCR (RT-PCR) of newborn mouse kidney total RNA. First, a pair of oligonucleotide primers (Gs and Gas; Table 1) that was designed on the highly conserved catalytic domain of murine (PTPϕ [23]; a murine macrophage cytoplasmic phosphatase) was used to generate a 351 bp fragment from the cDNA library. Taq polymerase and buffer conditions were the same as those recommended by the thermal cycler manufacturer (Perkin-Elmer Corp., Foster City, CA, USA), and a 30 cycle amplification (30 seconds at 94°C, 1 min at 55°C, and 2 min at 72°C) was carried out. The resulting PCR fragment was then used as the starting point to isolate a series of overlapping cDNA clones that spanned approximately 3.2 kb. All of the forward primers except G18s (Table 1) for these steps were derived from the corresponding human GLEPP1 sequence [19], and all of the reverse primers were generated from sequences derived from the new mouse-specific cDNA clones except G19as. The G18s and G19as primers were both derived from the partial GLEPP1 genomic DNA sequence (Table 1). The PCR conditions for these amplifications were set for 40 cycles (30 seconds at 94°C, 1 min at 60°C, and 2 min at 68°C). RT-PCR analysis of the transcripts was carried out with two pairs of primers (G18s and G13as and G11 and Gas; Table 1) using the Access RT-PCR system (Promega Corp., Madison, WI, USA). Following first-strand cDNA synthesis, the thermocycling program was set for 40 high-stringency cycles, as described previously in this article for the PCR conditions.

To obtain the initiation codon and 5′-untranslated re-

Table 1. Primers used for polymerase chain reaction (PCR) amplifications

Forward primers: (5′-3′)	
Gs	TAGACACTTCCGGATCAACTA (PTPϕ, nt. 1181–1201)
G1s	CCGTGCTGGCCATCCTTAGCACAC (human GLEPP1, nt. 2643–2666)
G7s	CAGCCCCAGTGGCTCCGGAAA (human GLEPP1, nt. 2061–2081)
G11s[b]	GGACCACCTTCAGATCCTGTGAC (human GLEPP1, nt. 1724–1746)
G18s[ab]	CCTTGCCTGTAACCAGTG (mouse GLEPP1, nt. 705–722)
λgt11s	GGTGGCGACGACTCCTGGAGCCCGTC (λgt11 vector, nt. 1033–1058)
Reverse primers: (5′-3′)	
Gas[b]	ATCAGCTGCACACACTGATG (PTPϕ, nt. 1512–1531)
G2as	CCGTGATCGGGCCAGGCTGTGTAG (mouse GLEPP1, nt. 3586–3609)
G3as	GCTCCTCTGTCTGTACCATTGACA (mouse GLEPP1, nt. 3825–3848)
G4as	CAAGGGTCACTAAGAGCAGTC (mouse GLEPP1, nt. 2886–2906)
G5as	GTTCAGTGGCAGATCTGCAGC (mouse GLEPP1, nt. 3164–3184)
G9as	GTGGGACAGGTCAATGGTGG (mouse GLEPP1, nt. 2349–2368)
G10as	GACAAGGCGGGGCAAGGAGG (mouse GLEPP1, nt. 2520–2539)
G13as[b]	GATAGGTCGGGTCCAGCTCAG (mouse GLEPP1, nt. 2024–2044)
G19as[a]	GAGGATATTGAAGCCATC (mouse GLEPP1, nt. 1457–1474)
G22as	CAGTAGCTGATGTTCACTCTGCTGAAC (mouse GLEPP1, nt. 799–825)
λgt11as	CAACTGGTAATGGTAGCGACCGGCGC (λgt11 vector, nt. 1082–1107)

[a]Primers were initially designed from the partial sequence of the mouse GLEPP1 genomic DNA (Wiggins et al; unpublished observations). Our data are consistent with these sequences. The nucleotide numbers referred to here come from the complete mouse GLEPP1 cDNA sequence. The accession number is AF135166.

[b]Primers were used for both PCR and RT-PCR

gion of the cDNA, a sense primer (λgt11s) was designed from the λgt11 vector, and a gene-specific antisense primer (G22as) was designed from the cloned mouse GLEPP1 sequences (Table 1). The cDNA was amplified with EX Taq DNA Polymerase (TaKaRa Shuzo Co., Ltd., Shiga, Japan) at 30 cycles (98°C for 20 seconds and 68°C for 1 min). The 3′ end sequence of mouse GLEPP1 was obtained using the sense primer G1s from the corresponding human GLEPP1 sequence, an antisense primer (λgt11as) from the λgt11 vector (Table 1), and 30 cycles of amplification (98°C for 20 seconds and 68°C for 4 min) were carried out.

DNA sequence analysis

Polymerase chain reaction products were purified and cloned into plasmids using a pGEM-T Easy Vector System (Promega), and inserts were sequenced on an ABI377 automated DNA sequencer. Sequence analyses and comparisons were accomplished with the Genetics Computer Group (GCG) programs [24] and the BLAST program from the National Library of Medicine/National Institutes of Health, the World Wide Web Prediction Server at the Center for Biological Sequence Analysis, Department of Biotechnology, the Technical University of Denmark (http://www.cbs.dtu.dk/services/SignalP/) and the United Kingdom Human Genome Mapping Project Resource Center (http://www.hgmp.mrc.ac.uk/GenomeWeb/).

Northern blot analysis

Samples containing approximately 40 μg total RNA from mouse kidney at various stages and six-week-old mouse brain were electrophoresed on 1.0% denaturing agarose gels and transferred onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). Blots were probed with a cDNA fragment corresponding to the 827 bp EcoR1 restriction product of mouse GLEPP1 (clone RG-51, bp 705 to 1531) that had been labeled with [α^{32}P] dCTP (Amersham Pharmacia Biotech) using a Prime-a-Gene Labeling System (Promega). To quantitate message expression among different samples, the amounts of loaded RNA were controlled for using a GAPDH probe as an internal standard, and autoradiographs were analyzed on a Phospho Image Quant Scanner (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

In situ hybridization

Newborn mouse kidneys were fixed with 4% paraformaldehyde at 4°C overnight, cryoprotected in 30% sucrose for an additional 24 hours, and embedded in OCT Compound at −20°C. Sections, 10 μm thick, were cut and mounted on precleaned glass slides (Fisher Scientific Co., Pittsburgh, PA, USA) and dried at 50°C for one to three minutes and at room temperature for one to two hours. Sections were postfixed in 4% (wt/vol) paraformaldehyde, pretreated with 0.2 mol/L HCl, proteolyzed by predigested pronase (0.16 mg/mL), and treated with 0.1 mol/L triethanolamine containing 0.25% (vol/vol) acetic anhydride and were dehydrated again. Riboprobes were prepared using the in vitro Transcription Systems (Promega), and the EcoRI fragment of clone RG-51 (nt 1532 to 2044 bp) was ligated into the pGEM-4Z plasmid (Promega). For the antisense probe, the plasmid was linearized by digestion with BamH1, and in vitro transcription was carried out with Sp6 RNA polymerase and [α^{33}P]-dUTP (100 μCi, 3000 Ci/mmol; Amersham Pharmacia Biotech). The same plasmid was linearized with BglI, blunted with Klenow reagent, and transcribed by T_7 RNA polymerase to generate the sense probe. Hybridizations with radiolabeled antisense or sense probes (5×10^6 cpm/slide) were carried out at 50°C overnight in hybridization buffer [0.25 mol/L NaCl, 0.01 mol/L Tris-HCl, 0.01 mol/L NaH_2PO_4, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (BSA), 50% formamide, 10 mmol/L dithiothreitol (DTT), 10% dextran sulfate, and 1 mg/mL yeast total RNA]. Sections were then treated with 20 mg/mL RNase A and washed in hybridization buffer containing 50% formamide at room temperature overnight. The next day, sections were dehydrated in a graded series of ethanol, air dried, dipped in emulsion (Ilford, Ltd., Cheshire, UK) and, after three to five days of exposure, were developed with Kodak D-19.

Immunofluorescence studies

Rat GLEPP1 fibronectin domains 2 through 5 were expressed as a GST fusion protein, and the glutathione-eluted affinity-purified fusion protein was used to immunize rabbits. An IgG fraction of immune serum was then immunopurified using the rat GLEPP1-GST fusion protein immobilized on cyanogen-activated Sepharose 4B beads (Amersham Pharmacia Biotech) and eluted using HCl-glycine, pH 2.5. Fresh, unfixed kidney samples from fetal and newborn mice were snap frozen in 2-methyl butane chilled in a dry ice-acetone bath and embedded in OCT compound (Miles, Elkhart, IN, USA). Sections approximately 4 to 6 μm thick were cut at −20°C in a cryostat and air dried at room temperature. Slides were double labeled with rat antimouse laminin mAb 5A2 (100 μg/mL) [25] and the affinity-purified rabbit anti-GLEPP1 IgG (30 μg/mL) [19]. Sections were then incubated with a mixture of rhodamine-conjugated goat anti-rat IgG (1:20) and fluorescein-conjugated goat antirabbit IgG (1:100; ICN Biomedicals, Costa Mesa, CA, USA). Some sections were also processed for anti-GLEPP1 labeling alone. Controls were labeled with preimmune IgGs.

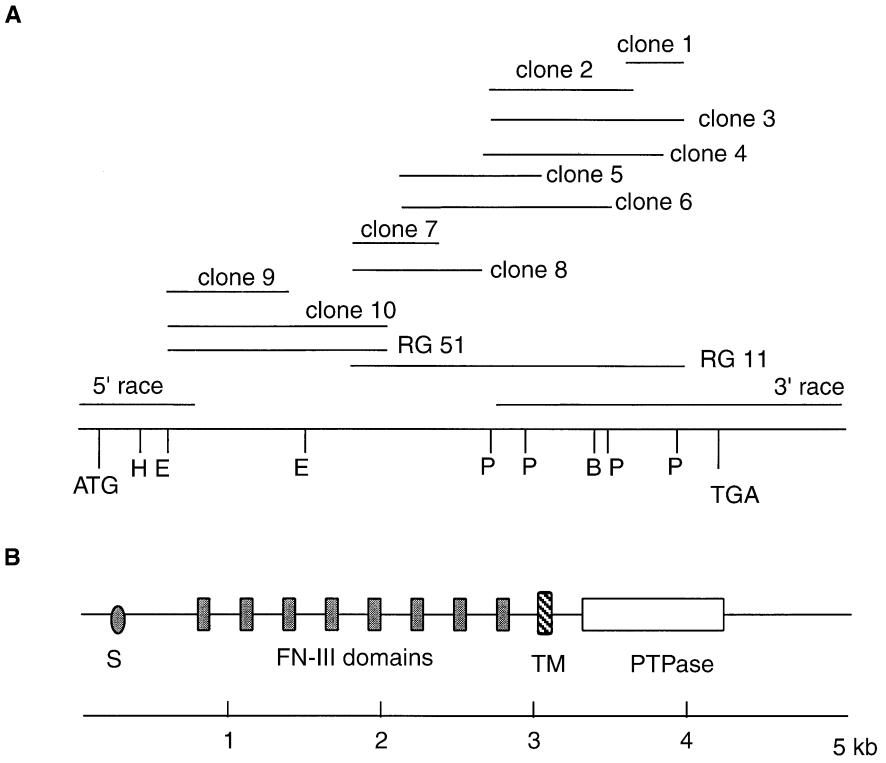

Fig. 1. Schematic structure of overlapping cDNA clones for mouse GLEPP1 and domain structure of the corresponding polypeptides. (*A*) Alignment of 14 overlapping cDNA clones and partial restriction map of the cDNA. The translation initiation signal (ATG) and translation stop codon (TAG) are indicated. Restriction sites are HindIII (H), EcoRI (E), PstI (P), and BglI (B). (*B*) Domain structure of mouse GLEPP1 showing signal sequence (S; grey oval), fibronectin type III-like repeats (■), transmembrane region (TM; ▨), and phosphatase catalytic domain (□).

RESULTS

Cloning and sequencing

The full-length cDNA for mouse GLEPP1 was cloned by PCR from a newborn mouse kidney λgt11 cDNA expression library and by RT-PCR from newborn mouse kidney total RNA. The sequences of the two overlapping RT-PCR clones corresponded completely with the 12 overlapping clones obtained by PCR (Fig. 1). The complete nucleotide and predicted amino acid sequences are shown in Figure 2. The full-length cDNA spans 5106 nucleotides, including a 352 base 5′ untranslated region, an open reading frame of 3594 nucleotides, and a 3′ untranslated region of 1160 bases.

The 3′ noncoding sequence contains a polyadenylation signal (AUUAAA) located 23 nucleotides upstream of the poly(A) tail. The open reading frame encodes a protein of 1198 amino acids with a predicted molecular mass of 135 kD and an isoelectric point of 5.57. The first in-frame ATG is in favorable context for initiation of translation [26]. Computer sequence analysis revealed that the homologies between mouse and human or rabbit GLEPP1 are approximately 80% identical at the nucleotide level and approximately 91% identical at the amino acid level, respectively. Primary amino acid sequence alignment between mouse and human GLEPP1 showed that the mouse protein has one additional amino acid at position 257 and another nine additional amino acid residues (284, 285, 286, 287, 288, 289, 290, 291, and 292) in the extracellular domain, for a total 1198 amino acids (Fig. 3). When compared with rabbit GLEPP1, the mouse has 11 additional amino acid residues at sequence positions 257 and 275 and 284 through 292 (Fig. 3).

Analysis of the deduced amino acid sequence of mouse GLEPP1 predicted that the signal peptide extends through the first 35 amino acids with a cleavage site following residues AVQ (Fig. 2). This putative signal sequence is also in agreement with the "−3−1" rule [27] and is six amino acids longer than that for human and rabbit GLEPP1. The mature mouse GLEPP1 protein is therefore predicted to consist of 1163 amino acid residues overall with a 794 amino acid extracellular domain (residues 36 to 829), a 25 amino acid-long transmembrane region (residues 830 to 854), and a cytoplasmic tail with a single catalytic PTPase domain (residues 899 to 1174; Fig. 2).

Human and rabbit GLEPP1 proteins have 16 and 15 N-glycosylation sites, respectively, and 14 of these are identical. Motif analysis showed that mouse GLEPP1 has 15 putative N-glycosylation sites, but 5 of these differ from those in human and rabbit. These are located at N-279, N-288, N-297, N-380, and N-722 (Fig. 2). The putative glycosaminoglycan binding site (amino acids 685 to 690) in mouse GLEPP1 (RKKIKK) differs only by a single conservative substitution from the human and rabbit sequences (KKKIKK). Based on predicted secondary structure and homology to the human and rabbit

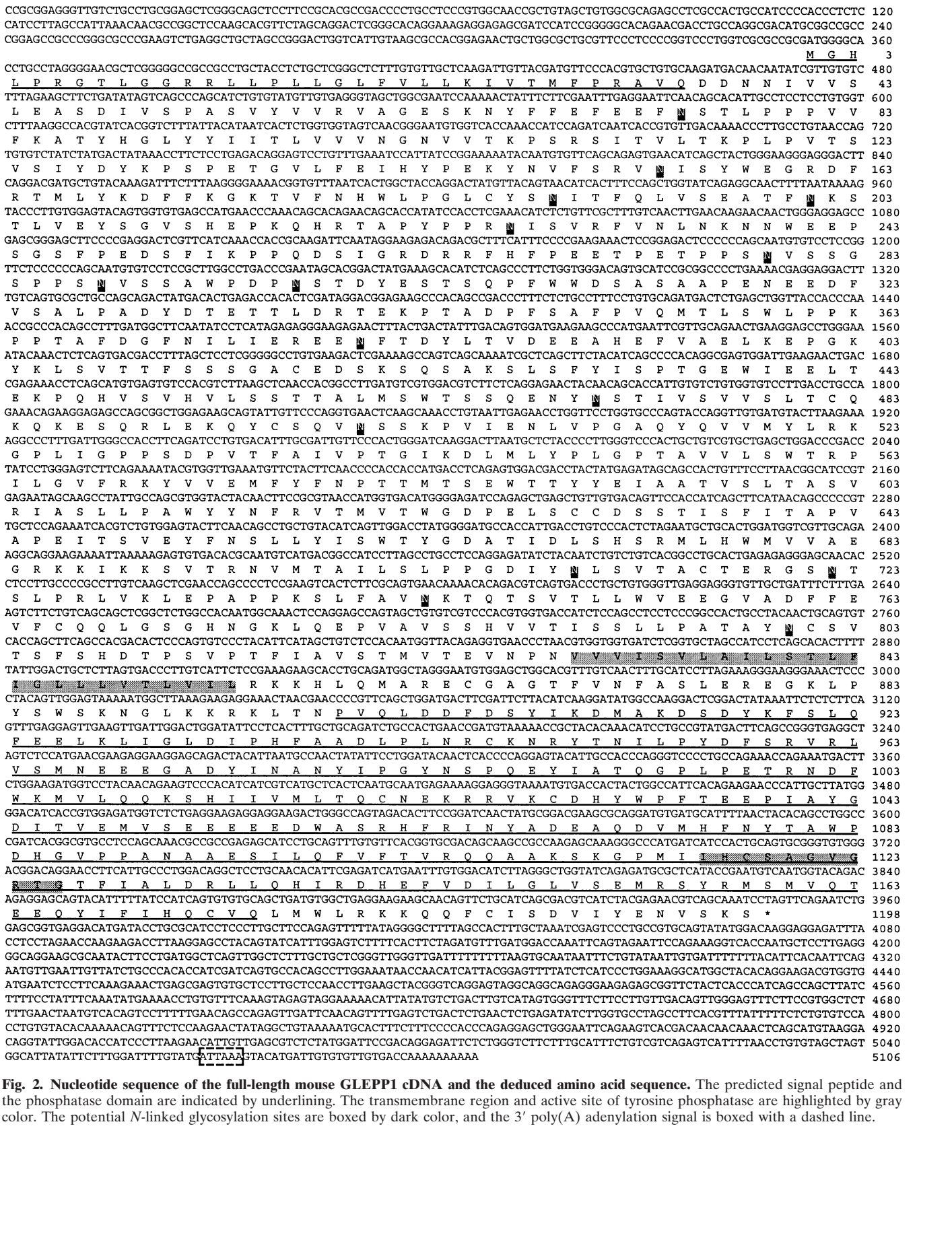

Fig. 2. Nucleotide sequence of the full-length mouse GLEPP1 cDNA and the deduced amino acid sequence. The predicted signal peptide and the phosphatase domain are indicated by underlining. The transmembrane region and active site of tyrosine phosphatase are highlighted by gray color. The potential *N*-linked glycosylation sites are boxed by dark color, and the 3′ poly(A) adenylation signal is boxed with a dashed line.

```
          30                                                                                                  129
  hGlepp1 fhvtvq---n ~---------v i---------k it-------- ---------- ---i---s-- ---------- ---------- ---------- ----------
 rbGlepp1 fhvtvr---s ---------v i---------k it-------- ---------- ---i---n-- ---------- ---------- ---------- ----------
  mGlepp1 ~~~~~~~~n ---------i v---------r va-------- ---------- ---v---t-- ---------- ---------- ---------- ----------
Consensus ------DDN- IVVSLEASD- -SPASVYVV- --GESKNYFF EFEEFNSTLP PPV-FKA-YH GLYYIITLVV VNGNVVTKPS RSITVLTKPL PVTSVSIYDY
          130                                                                                                 229
  hGlepp1 ---------- ---------- -t-------- -kd------- ---------- -----m---- ---------- ---------- ---------- ------q---
 rbGlepp1 ---------- ---------- -t-------- -ka------- ---------- -----i---- ---------- ---------- ---------- ------r---
  mGlepp1 ---------- ---------- -s-------- -rd------- ---------- -----l---- ---------- ---------- ---------- ------r---
Consensus KPSPETGVLF EIHYPEKYNV F-RVNISYWE G--FRTMLYK DFFKGKTVFN HWLPG-CYSN ITFQLVSEAT FNKSTLVEYS GVSHEPKQHR TAPYPP-NIS
          230                                                                                                 329
  hGlepp1 --i------- ---q--n--- e--mrsq.dt -gkekl---t -----i-sg- i---...... ...---f--s ---t----y- ------a--s -de---v--m
 rbGlepp1 --i------- ---q--s--- e--mrsp.et -ekdri---t -----.-sg- i---...... ...---f--s ---t----y- ------t--s -de---v--m
  mGlepp1 --f------- ---p--s--- d--ikppqds -grdrr---p -----tppsn vss-sppsnv ssa---p--t ---s----f- ------a--n -ed---a--a
Consensus VR-VNLNKNN WEE-SG-FPE -SF------- I-----FHF- EETPE-P--N -SSG------ ---WPD-NS- DYE-TSQP-W WDSASA-PE- E--FVS-LP-
          330                                                                                                 429
  hGlepp1 e-enns--se t--s-sgs-- f-----i-t- ---------- --h-h----- ---e-lm--- ---------- ---------- ---s--s--t rk--------
 rbGlepp1 e-ennn--se a--p-pap-- f-----i-s- ---------- --h-h----- ---e-st--- ---------- ---------- ---a--s--t re--------
  mGlepp1 d-dtet--dr t--p-adp-- a-----t-s- ---------- --n-l----- ---d-lt--- ---------- ---------- ---s--a--d sk--------
Consensus -Y----TL-- -EK-T---FS -FPVQM-L-W LPPKPPTAFD GF-I-IEREE NFT-Y--VDE EAHEFVAELK EPGKYKLSVT TFS-SG-CE- --SQSAKSLS
          430                                                                                                 529
  hGlepp1 -----s---- ---------- ---------- ---------- ---------- ---------- -------t-- ----pi---- ---------i ----------
 rbGlepp1 -----t---- ---------- ---------- ---------- ---------- ---------- -------t-- ----ri---- ---------m ----------
  mGlepp1 -----t---- ---------- ---------- ---------- ---------- ---------- -------s-- ----pv---- ---------m ----------
Consensus FYISP-GEWI EELTEKPQHV SVHVLSSTTA LMSWTSSQEN YNSTIVSVVS LTCQKQKESQ RLEKQYC-QV NSSK--IENL VPGAQYQVV- YLRKGPLIGP
          530                                                                                                 629
  hGlepp1 ---------- ---------- ---------- ----y----- ---------- -a-------- ---------- -------n-- ---------- ----------
 rbGlepp1 ---------- ---------- ---------- ----y----- ---------- -a-------- ---------- -------n-- ---------- ----------
  mGlepp1 ---------- ---------- ---------- ----i----- ---------- -t-------- ---------- -------s-- ---------- ----------
Consensus PSDPVTFAIV PTGIKDLMLY PLGPTAVVLS WTRP-LGVFR KYVVEMFYFN P-TMTSEWTT YYEIAATVSL TASVRIA-LL PAWYYNFRVT MVTWGDPELS
          630                                                                                                 729
  hGlepp1 ---------- ---------- ---------- ------d-t- ---------- -----k---- ---------- ---------- ---------- -----ml---
 rbGlepp1 ---------- ---------- ---------- ------d-t- ---------- -----k---- ---------- ---------- ---------- -----ml---
  mGlepp1 ---------- ---------- ---------- ------a-i- ---------- -----r---- ---------- ---------- ---------- -----lp---
Consensus CCDSSTISFI TAPVAPEITS VEYFNSLLYI SWTYGD-T-D LSHSRMLHWM VVAEG-KKIK KSVTRNVMTA ILSLPPGDIY NLSVTACTER GSNTS--RLV
          730                                                                                                 829
  hGlepp1 ---------- ---------- ---------- ---------v --sqkt---- ---------- ---------- ---------- s---------- ------m---
 rbGlepp1 ---------- ---------- ---------- ---------v --glet---- ---------- ---------- ---------- s---------- ------m---
  mGlepp1 ---------- ---------- ---------- ---------l --ghng---- ---------- ---------- ---------- t---------- ------v---
Consensus KLEPAPPKSL FAVNKTQTSV TLLWVEEGVA DFFEVFCQQ- GS----KLQE PVAVSSHVVT ISSLLPATAY NCSVTSFSHD -PSVPTFIAV STMVTE-NPN
```

Fig. 3. Comparison of mouse GLEPP1 (mGLEPP1) extracellular domain with those of human (h) and rabbit (r) GLEPP1. Identical amino acids are shown on the consensus line, and differences among the three proteins are displayed. The extra amino acids in mouse GLEPP1 are highlighted, and the potential duplication peptides are underlined.

GLEPP1, mouse GLEPP1 also has eight fibronectin type III repeats in the extracellular domain [18, 19, 28]. An alignment of the extracellular domains between human, rabbit, and mouse GLEPP1 is shown in Figure 3, where the identical amino acids among these three GLEPP1 sequences are shown on the consensus line. The nine extra amino acids in mouse GLEPP1 (SPPSNVSSA), which have no homology with any other known tyrosine phosphatase, are inserted near the middle of the third fibronectin III repeat and may represent a duplication of a contiguous segment of DNA that encodes PPSNVSS (Fig. 3).

We also compared the primary structure of the catalytic domain of mouse GLEPP1 with several other RPTPs. As shown in Table 2, the phosphatase domain of mouse GLEPP1 is homologous to human and rabbit GLEPP1 but contains highest homologies with RPTP-BK, a rat RPTP previously found to be restricted to brain and kidney [29]. However, a comparison of mouse GLEPP1 with PTPϕ [30] indicated that these two proteins shared the same phosphatase domain (99.6% identity). Motif analysis also predicted that the mouse GLEPP1 cytoplasmic domain contains four sites of casein kinase II phosphorylation, which are the same as those seen in human and rabbit and located at S-965, S-985, T-999, and S-1051. In addition to the protein kinase C phosphorylation site of S-1155, which is identical

Table 2. Amino acid sequence identity (%) of mouse GLEPP1 versus other related PTP molecules

	Entire length	Extracellular domain	PTPase domain
PTPϕ	99.4	—	99.6
RPTP-BK	95.5	94.4	98.6
GLEPP1 (human)	91.0	89.1	96.0
GLEPP1 (rabbit)	90.8	88.9	95.3
HPTPβ	30.8	23.4	56.4
DPTP10D	30.0	23.0	53.0
rPTP-GMC1	28.1	27.1	47.6

GenBank accession numbers of citation sequences are: AF135166 (GLEPP1, mouse), U37465 (PTPϕ), U28938 (RPTP-BK), U20489 (GLEPP1, human), U09490 (GLEPP1, rabbit), X54131 (HPTPβ), M80538 (DPTP10D), AF063249 (rPTP-GMC1).

A

```
          1                                                  50
 hGlepp1  PVQLDDFDAY IKDMAKDSDY KFSLQFEELK LIGLDIPHFA ADLPLNRCKN
rbGlepp1  PVQLDDFDAY IKDMAKDSDY KFSLQFEELK LIGLDIPHFA ADLPLNRCKN
  PTPphi  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~NRCKN
 mGlepp1  PVQLDDFDSY IKDMAKDSDY KFSLQFEELK LIGLDIPHFA ADLPLNRCKN
 RPTP-BK  PVQLDDFDSY IKDMAKDSDY KFSLQFEELK LIGLDIPHFA ADLPLNRCKN
          51                                                 100
 hGlepp1  RYTNILPYDF SRVRLVSMNE EEGADYINAN YIPGYNSPQE YIATQGPLPE
rbGlepp1  RYTNILPYDF SRVRLISMNE EEGADYINAN YIPGYNSPQE YIATQGPLPE
  PTPphi  RYTNILPYDF SRVRLVSMNE EEGADYINAN YIPGYNSPQE YIATQGPLPE
 mGlepp1  RYTNILPYDF SRVRLVSMNE EEGADYINAN YIPGYNSPQE YIATQGPLPE
 RPTP-BK  RYTNILPYDF SRVRLVSMNE EEGADYINAN YIPGYNSPQE YIATQGPLPE
          101                                                150
 hGlepp1  TRNDFWKMVL QQKSQIIVML TQCNEKRRVK CDHYWPFTEE PIAYGDITVE
rbGlepp1  TRNDFWKMVL QQKSQMIVML TQCNEKRRVK CDHYWPFTEE PIAYGDITVE
  PTPphi  TRNDFWKMVL QQKSHIIVML TQCNEKRRVK CDHYWPFTEE PIAYGDITVE
 mGlepp1  TRNDFWKMVL QQKSHIIVML TQCNEKRRVK CDHYWPFTEE PIAYGDITVE
 RPTP-BK  TRNDFWKMVL QQKSRIIVML TQCNEKRRVK CDHYWPFTEE PIAYGDITVE
          151                                                200
 hGlepp1  MISEEEQDDW ACRHFRINYA DEMQDVMHFN YTAWPDHGVP TANAAESILQ
rbGlepp1  MISEEEQDDW AHRHFRINYA DEMQDVMHFN YTAWPDHGVP TANAAESILQ
  PTPphi  MVSEEEEEDW ASRHFRINYA DEAQDVMHFN YTGWPDHGVP PANAAESILQ
 mGlepp1  MVSEEEEEDW ASRHFRINYA DEAQDVMHFN YTAWPDHGVP PANAAESILQ
 RPTP-BK  MVSEEEQEDW ASRHFRINYA DEAQDVMHFN YTAWPDHGVP PANAAESILQ
          201                                                250
 hGlepp1  FVHMVRQQAT KSKGPMIIHC SAGVGRTGTF IALDRLLQHI RDHEFVDILG
rbGlepp1  FVHMVRQQAT KSKGPMIIHC SAGVGRTGTF IALDRLLQHI RDHEFVDILG
  PTPphi  FVFTVRQQAA KSKGPMIIHC SAGVGRTGTF IALDRLLQHI RDHEFVDILG
 mGlepp1  FVFTVRQQAA KSKGPMIIHC SAGVGRTGTF IALDRLLQHI RDHEFVDILG
 RPTP-BK  FVYTVRQQAT KSKGPMIIHC SAGVGRTGTF IALDRLLQHI RDHEFVDILG
          251                      278
 hGlepp1  LVSEMRSYRM SMVQTEEQYI FIHQCV~~
rbGlepp1  LVSEMRSYRM SMVQTEEQYI FIHQCV~~
  PTPphi  LVSEMRSYRM SMVQTEEQYI FIHQCVQL
 mGlepp1  LVSEMRSYRM SMVQTEEQYI FIHQCV~~
 RPTP-BK  LVSEMRSYRM SMVQTEEQYI FIHQCV~~
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B

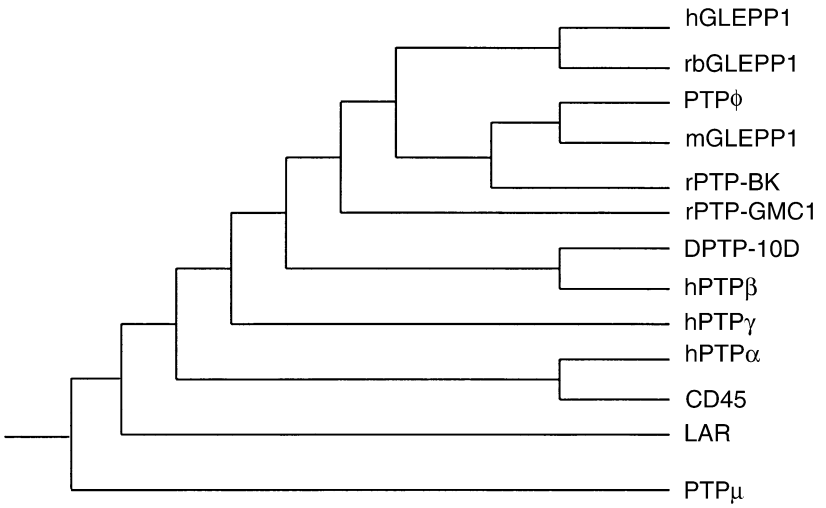

Fig. 4. (*A*) The phosphatase domain of mouse GLEPP1 (mGLEPP1) and other highly homologous PTPases [PTPϕ, human (h) and rabbit (r) GLEPP1, and RPTP-BK] were aligned by PILEUP in GCG. Shaded boxes highlight diversities. (*B*) A possible phylogenetic tree of some PTPase gene family members. Genetic distances were calculated based on the sequences of the core PTPase domains. GenBank accession numbers are as follows: hGLEPP1 (U20489), rbGLEPP1 (U09490), PTPϕ (U37465), mGLEPP1 (AF135166), rPTP-BK (U28938), rPTP-GMC1 (AF063249), DPTP-10D (M80538), hPTPβ (X54131), hPTPα (CAA38067), hPTPγ (CAA38065), CD45 (P06800), LAR (S46216), and PTPμ (P28828). The tree was constructed using the GROWTREE program in GCG.

to that in human and rabbit, mouse GLEPP1 has an additional site at T-1102. The putative cAMP- and cGMP-dependent protein kinase phosphorylation site (RKLT) is located intracellularly at residues 894 to 897, but is not within the PTPase domain. The 11 amino acid tyrosine-specific protein phosphatase active site (IHCSAGVGRTG) is found at residues 1116 to 1126.

The homologies over entire lengths, ectodomains, and catalytic domains between mouse, human, rabbit, several other RPTPs, and PTPϕ are shown in Table 2. When sequences encoding the catalytic domains of those phosphatases with the highest degrees of homologies were compared, an extremely high degree of identity was seen (Fig. 4A). To analyze the molecular evolution of mouse

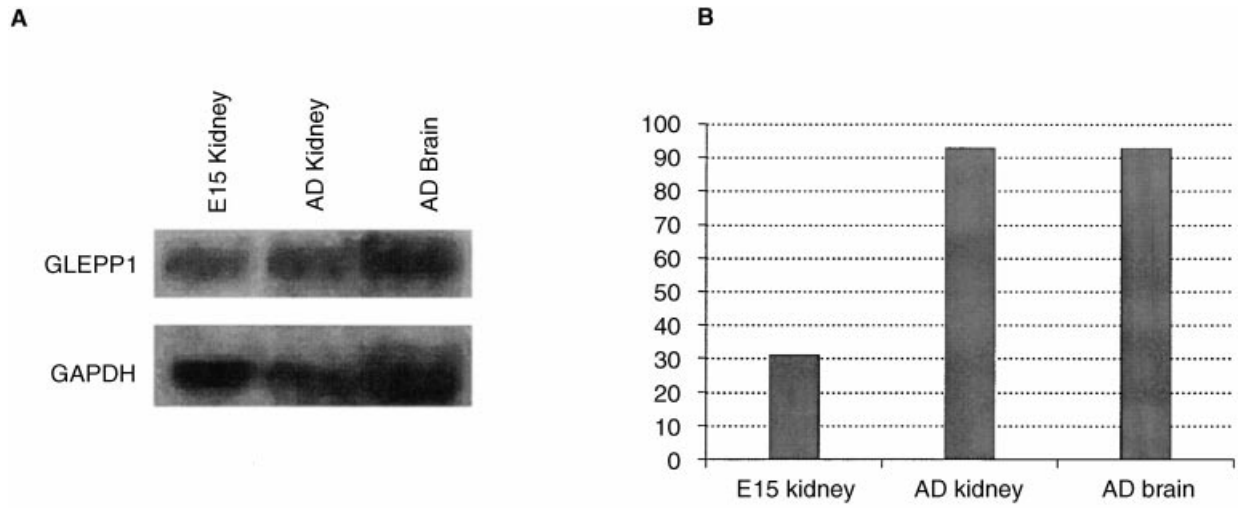

Fig. 5. Northern blot analysis of GLEPP1 expression in E15 and adult (AD) kidney and adult (AD) brain. (*A*) Blot showing a major ~5.5 kb band detected at both mouse kidney samples and in adult brain. The same blot was stripped and rehybridized with a GAPDH cDNA probe as a loading control (lower panel). (*B*) Phosphorimager histogram showing relative intensity of GLEPP1 hybridization signals. The expression of GLEPP1 mRNA in adult kidney and brain is approximately threefold greater than that seen in embryonic day 15 (E15) kidney.

GLEPP1 among the RPTP family, the entire amino acid sequences of different types of phosphatases were aligned (Table 2), and genetic distances were calculated to create a phylogenetic tree (Fig. 4B). These data indicate that mouse GLEPP1 has its closest evolutionary relationship to PTPϕ and RPTP-BK.

Although the total nucleotide sequence of mouse GLEPP1 is shorter than that for human and rabbit (by 309 bp and 576 bp, respectively), the 5′-untranslated region of mouse GLEPP1 is approximately 170 bp longer. Additionally, in the 5′-untranslated regions, there are comparatively large diversities between mouse and human (53% identity) and mouse and rabbit (63% identity).

Expression and distribution

Northern blot analysis of E15 and adult mouse kidney total RNA revealed that the mouse GLEPP1 gene was expressed as a single transcript of approximately 5.5 kb (Fig. 5A), which corresponds to the cDNA obtained. Based on phosphorimager analysis, the level of GLEPP1 transcription in adult mouse kidney was approximately threefold higher than that seen in the E15 kidney (Fig. 5B). Our results also showed that GLEPP1 mRNA was expressed in mouse brain, which is in agreement with the restricted expression patterns of human and rabbit GLEPP1, and RPTP-BK [29]. By Northern analysis, however, we found transcription levels of GLEPP1 in six-week-old mouse brain that were approximately the same as those seen in six-week-old kidney (Fig. 5B). This differs from the transcription of RPTP-BK in rats, which is down-regulated during brain development, and only trace amounts of message are detected in rat brain beyond 14 days of age [29].

To study the renal expression of mouse GLEPP1 mRNA in more detail, we carried out in situ hybridization on newborn mouse kidney sections using ^{33}P-labeled antisense riboprobes specific for mouse GLEPP1. Beginning with the comma-shaped stage of nephron development and continuing through the S-shaped stage, a low but highly specific hybridization signal was detected exclusively over visceral epithelial cells (developing podocytes) of early glomeruli (Fig. 6). Developing endothelial/mesangial cells in vascular clefts, as well as epithelial cells of other segments within the nephric figures, were unlabeled (Fig. 6). Additionally, as glomerular development progressed to the capillary loop stage, the intensity of the hybridization signals over podocytes increased (Fig. 6), and maturing stage glomeruli located deeper within the cortex were heavily labeled. GLEPP1 mRNA was not identified in any other kidney epithelial or mesenchymal cells. Likewise, when sections of newborn kidney were labeled with sense riboprobes as controls, no hybridization was detected on podocytes of capillary loop and maturing stage glomeruli or elsewhere in the tissue.

To evaluate the distribution of GLEPP1 protein during kidney development, we examined sections from E12 to E14 and newborn kidneys that had been double-labeled with anti-GLEPP1 and antilaminin antibodies to identify locations of podocytes clearly at all stages of differentiation. GLEPP1 protein was not observed in E12 and E13 kidneys and was first detected at E14, where weak labeling was seen chiefly on apical and lateral cell surfaces of podocytes of capillary loop stage glomeruli

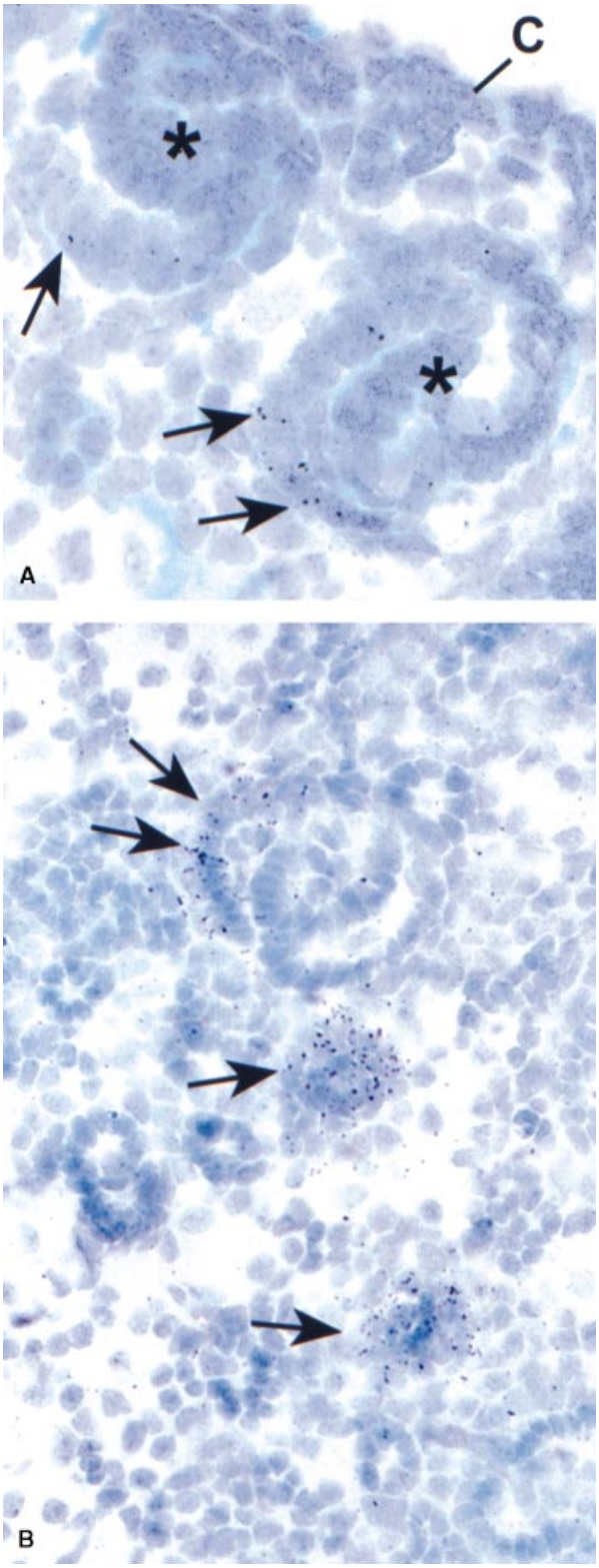

Fig. 6. In situ hybridization of newborn mouse kidney with ^{33}P-labeled GLEPP1 riboprobes. (*A*) Early nephric figures containing hybridization signals only over visceral epithelial cells (arrows). Developing tubular epithelial cells (*) and all other cells are negative (C is capsule). (*B*) Deeper in the cortex, developing podocytes of early capillary loop stage glomeruli contain stronger hybridization signals (arrows). Note complete absence of labeling elsewhere.

(Fig. 7). The visceral epithelial cells of earlier staged comma- and S-shaped nephric figures, which labeled with antisense riboprobes by in situ hybridization (Fig. 6), were negative by immunofluorescence (Fig. 7). In newborn kidneys, GLEPP1 protein was relatively much more abundant than that seen at E14, but was again confined to podocytes of capillary loop stage and more mature glomeruli (Fig. 8). Early nephric figures developing immediately beneath the newborn kidney capsule were negative (Fig. 8).

DISCUSSION

Glomerular capillaries are comprised of highly differentiated endothelial cells, the glomerular basement membrane, and epithelial podocytes, which together constitute the plasma filtration barrier. Early in glomerular development, immature podocytes are tall cuboidal/columnar cells that are joined by apical tight junctional complexes. During podocyte differentiation, the apical tight junctions migrate basally alongside interdigitating cytoplasmic extensions that are forming the foot processes or pedicels [31]. As foot process interdigitation proceeds in capillary loop stage glomeruli, the tight junctions between podocytes disappear and by unknown mechanisms are replaced by slit diaphragms. At this time in podocyte development, there is also the abrupt appearance of the protein nephrin within the filtration slits, exactly at the site of foot process apposition [32], which is consistent with nephrin constituting a major structural element of the slit diaphragm [33]. Because earlier studies with anti-GLEPP1 antibodies localized the RPTP exclusively on podocyte cell surfaces of human and rabbit kidneys, we were motivated to clone the cDNA for murine GLEPP1 and examine its expression during podocyte development.

As expected, cDNA and amino acid sequence comparisons showed strong homologies between mouse, human, and rabbit GLEPP1 (approximately 91%). Surprisingly, mouse GLEPP1 had even higher overall homologies (95.5%) with rat RPTP-BK and shared an almost identical (99.4%) amino acid sequence for the cytoplasmic phosphatase domain with murine PTPϕ. Three isoforms of PTPϕ were originally identified from a mouse macrophage cDNA library and were thought to arise by alternative splicing [30]. Two of these have short extracellular domains with identical membrane-spanning regions and are designated as the long-insert (47 kD) and short-insert (43 kD) isoforms, respectively. A third, no-insert variant with an identical catalytic sequence but lacking the transmembrane domain was predicted to be a cytosolic PTP. Although Northern analysis showed expression of approximately 2.8 kb bands (which would include all three PTPϕ isoforms) in mouse bone marrow, lung, spleen, thymus, and heart, a much larger approximately 5.5 kb

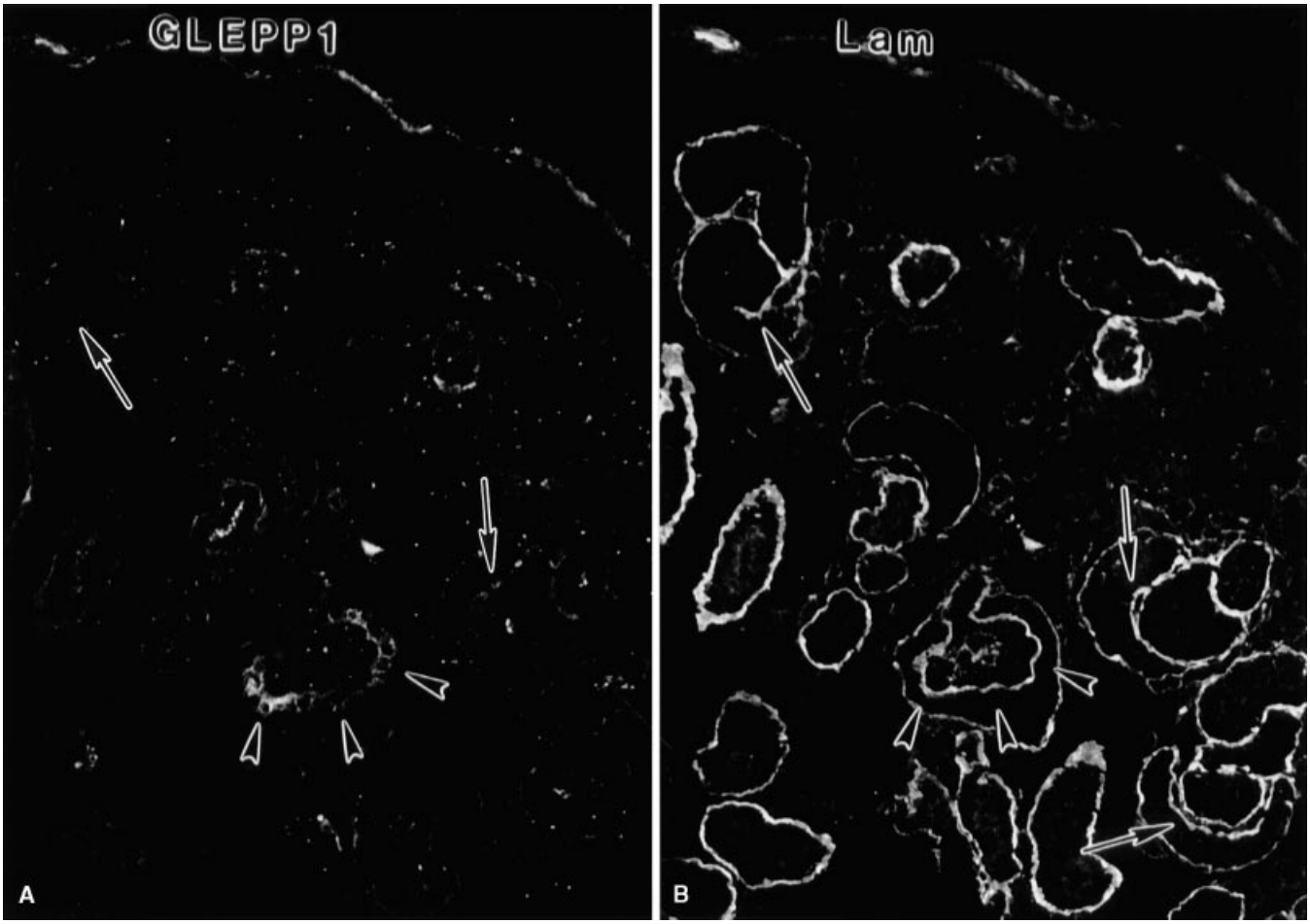

Fig. 7. Cryostat section of embryonic day 14 (E14) mouse kidney cortex doubly labeled with anti-GLEPP1 (*A*) and antilaminin IgGs (*B*). (A) GLEPP1 is first detected on developing podocytes of early capillary loop stage glomeruli (arrowheads). (B) The use of antilaminin IgG clearly outlines all nephric figures in field. Symbols are: arrowheads, capillary loop stage glomerulus; arrows, earlier staged comma- and S-shaped nephric figures. GLEPP1 protein is not yet detected on visceral epithelial cells in the comma- and S-shaped structures (compare A and B).

mRNA was seen in brain and kidney, suggesting the presence of a fourth isoform of PTPϕ [30]. The sequencing of the full-length mouse kidney approximately 5.1 kb GLEPP1 cDNA reported here with a cytoplasmic domain virtually identical to that for PTPϕ therefore appears to have confirmed this possibility.

RPTP-BK, also approximately 5.6 kb, was originally isolated from rat central nervous system and named for its restricted expression in brain and kidney [29]. Sequence comparisons of mouse GLEPP1, RPTP-BK, and PTPϕ showed that GLEPP1 contains the short insert isoform of PTPϕ, whereas RPTP-BK contains the insert found in the long PTPϕ isoform. These results showing the expression of two such closely related RPTPs restricted to the brain and kidney raise interesting biological questions on phosphatase diversity. RPTP-BK has been shown to have its most abundant expression in regions of the developing rat brain where neuronal axonogenesis is taking place and that expression levels in the brain diminish sharply at postnatal day 14 [29]. By contrast, RPTP-BK is not developmentally regulated in the kidney, where it is expressed constitutively in both newborn and mature glomeruli [29]. Our data on the expression of GLEPP1 in E15 and mature kidney, however, showed that a threefold up-regulation occurred with maturation, and a similarly high level of GLEPP1 mRNA was found in adult brain. Although we did not examine the expression of GLEPP1 in the brain in detail, its sustained synthesis there suggests that it may compensate or substitute for RPTP-BK in the adult. On the other hand, because both GLEPP1 and RPTP-BK are present in mature kidney, perhaps their PTPase catalytic activities are modulated by ligands that bind differentially to the slightly divergent extracellular segments displayed by these receptors.

The results of our immunolocalization results showed that the onset of GLEPP1 protein expression in the mouse kidney begins at E14. At that age, podocytes of

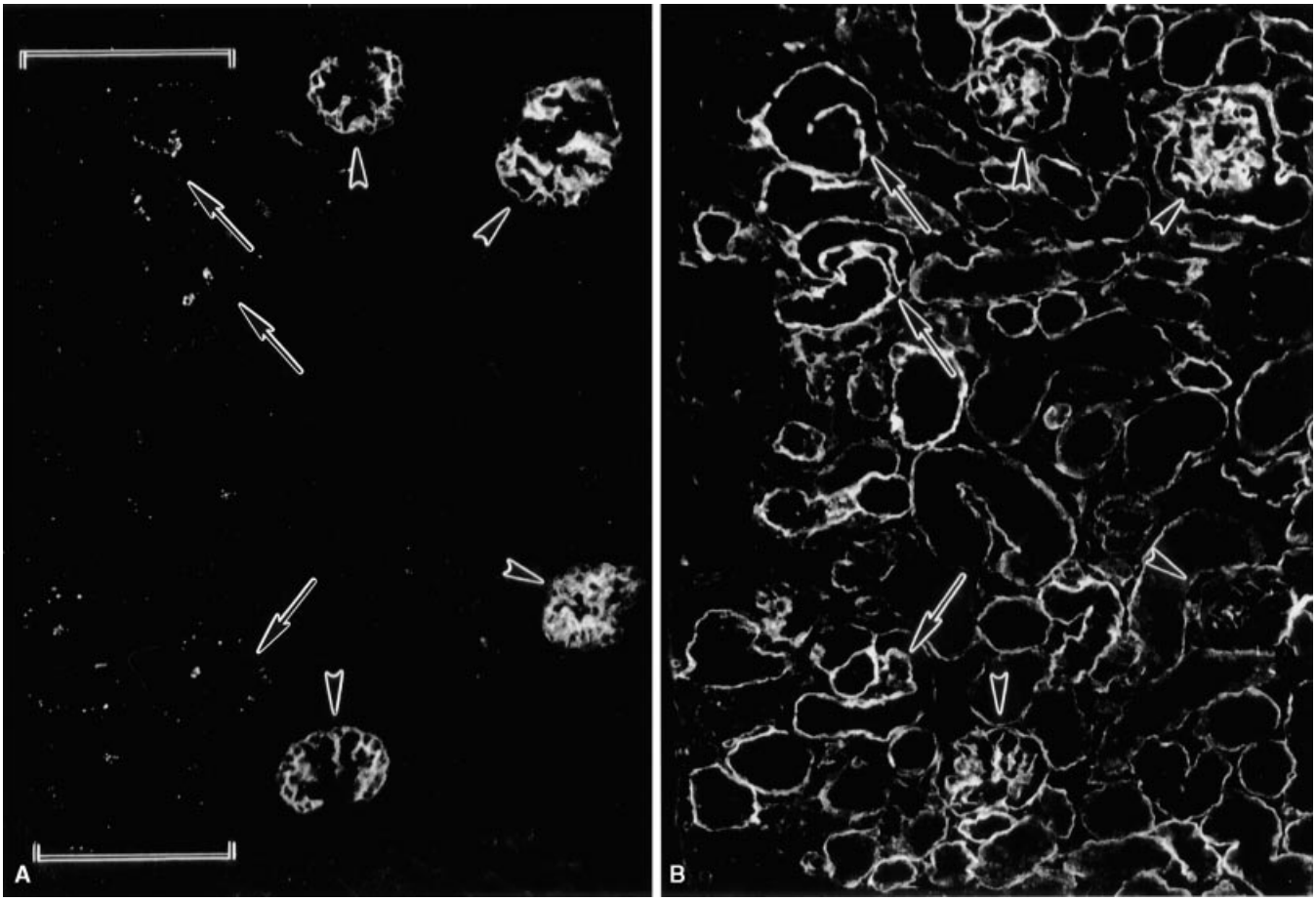

Fig. 8. Newborn mouse kidney cortex doubly labeled with anti-GLEPP1 (*A*) and antilaminin IgGs (*B*). (A) Anti-GLEPP1 only binds to developing podocytes of capillary loop and maturing stage glomeruli (arrowheads). The nephrogenic zone beneath the capsule (brackets) is negative. (B) Antilaminin identifies comma- and S-shaped figures whose visceral epithelial cells (arrows) do not yet express GLEPP1 (examine corresponding areas on left panel). Arrowheads point to the capillary loop and maturing glomeruli.

capillary loop stage glomeruli were labeled, but those of earlier comma- and S-shaped nephric figures were not. However, our in situ hybridization experiments clearly indicated that GLEPP1 mRNA could be detected in nascent podocytes of the earlier staged comma- and S-shaped nephric figures and that message levels increased markedly in capillary loop and maturing stage glomeruli. These findings are in general agreement with studies that immunolocalized GLEPP1 protein in fetal human [34] and newborn rabbit [20] kidneys, and all of the available data show that from the glomerular capillary loop stage to full glomerular maturation, GLEPP1 expression intensifies on and is restricted to podocytes exclusively. This pattern of GLEPP1 expression coincides with the elaboration of podocyte foot processes and the appearance of a number of other proteins likely to be crucially important for the acquisition of the unique podocyte phenotype.

Nephrin, which is the protein product of the *NPHS1* gene that is mutated in congenital nephrotic syndrome of the Finnish type [35], exhibits a temporal expression pattern in the mouse that is remarkably similar to that seen for GLEPP1. Both proteins, which in the mature kidney are expressed exclusively by podocytes, are first seen during foot process formation. Whereas GLEPP1 is located diffusely on podocyte cell surfaces, nephrin is restricted to intercellular zones (slit pores) between developing foot processes and becomes associated with the slit diaphragms [32]. Similarly, ZO-1 first appears in the apical tight junctions of visceral epithelial cells of comma- and S-shaped nephric figures and, as foot processes develop, becomes restricted to the cytoplasmic faces of the slit diaphragms [36]. Finally, podocalyxin, which is an approximately 140 kD sialoglycoprotein imparting a high net negative charge to the podocyte plasma membrane, is first seen on apical plasma membranes of developing podocytes in comma- and S-shaped stages and then is expressed on apical surfaces of foot processes [37]. Whether GLEPP1 interacts directly or indirectly with any of these proteins within podocytes in

vivo is not yet established. Nevertheless, treatment of epithelial monolayers with peroxovanadate, a phosphatase inhibitor, results in increased tyrosine phosphorylation and redistribution of the intercellular junctional proteins, ZO-1, ZO-2, E-cadherin, and catenins and also disrupts intercellular permeability barriers [38–40]. This evidence, along with a reduction of GLEPP1 observed on podocyte surfaces in minimal-change nephropathy, congenital nephrotic syndrome of the Finnish type, and focal segmental glomerulosclerosis [20], all implicate this RPTP as a mediator of podocyte differentiation and maintenance. The molecular cloning of mouse GLEPP1 reported here should provide some additional tools to help explore the biological functions of this highly restricted and conserved phosphatase.

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